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Development and evaluation of oxaliplatin and irinotecan co-loaded liposomes for enhanced colorectal cancer therapy

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ABSTRACT

Drug combinations are widely employed in chemotherapy for colorectal cancer treatment. However, traditional cocktail combination in clinic causes the uncertainty of the treatment, owing to varying pharmacokinetics of different drugs. The aim of this study was to design co-loaded liposomes to achieve the synchronised delivery and release. Oxaliplatin and irinotecan hydrochloride, as one of recommended combination schemes for the treatment of colorectal cancer in clinic, were co-loaded into the liposomes. The particle sizes of the liposomes were <200 nm with uniform size distribution. *In vitro* release study showed that both drugs could be synchronously released from the liposomes, which means the optimized synergistic ratio of two drugs could be achieved. *In vitro* cellular uptake revealed that co-loaded liposomes could efficiently deliver different drugs into the same cells, indicating their potential as carriers for enhancing the cancer therapy. CLSM images of cryo-sections for *in vivo* co-delivery study also revealed that co-loaded liposomes had superior ability to co-deliver both the cargoes into the same tumor cells. Besides, *in vivo* NIRF imaging indicated that the liposomes could increase the drug accumulation in tumor compared with free drug. *In vitro* cytotoxicity evaluation demonstrated that co-loaded liposomes exhibited higher cytotoxicity than the mixture of single loaded liposomes in both CT-26 and HCT-116 cells. Furthermore, co-loaded liposomes also presented superior anti-tumor activity in CT-26 bearing BALB/c mice. *In vivo* safety assessment demonstrated that liposomes had lower toxicities than their solution formulations. These results indicated that oxaliplatin and irinotecan hydrochloride co-loaded liposomes would be an efficient formulation for improving colorectal cancer therapy with potential clinical applications.

1. Introduction

The incidence of colorectal cancer ranks third in cancers and is showing an increasing trend [1]. Chemotherapy is still one of the key approaches for the treatment of colorectal cancer. There are several chemotherapeutic drugs used in clinic, including fluorouracil, oxaliplatin, irinotecan hydrochloride and capecitabine. However, the emergence of drug resistance and tumor recurrence is often associated with the single drug based cancer chemotherapy, mainly due to pathway overlapping, cross-talk and neutralizing response that commonly occur with cancer monotherapy [2]. Because of limited use of single drug chemotherapy, drug combination therapy is quite popular in clinics. For example, oxaliplatin and irinotecan hydrochloride (IROX) combination has been recommended as one of first-line treatment schemes as per the guidelines of NCCN (National Comprehensive Cancer Network). The dosages are 85 mg/m^2 for oxaliplatin and 200 mg/m^2 for irinotecan hydrochloride, respectively [3].

Oxaliplatin is a third generation platinum-based cytotoxic drug, which acts by damaging the structure of DNA [4]. Irinotecan hydrochloride is a water soluble camptothecin category drug, and it acts as DNA topoisomerase I inhibitor [5]. The combination of the two drugs acts on DNA with different mechanisms, hence improved anti-tumor activity.

However, traditional combination in clinical use is just a cocktail [6]. Administration of a combination of drugs would be challenging because of the inability to control the dosage regimen and effective drug ratio at the target tissue, due to the differences in pharmacokinetics of individual drugs [7–9]. To overcome these limitations, it is critical to co-deliver two drugs into the tumor cells synchronously [5,10,11].

Nanoscale drug delivery systems provide an opportunity of co-delivering different drugs and many co-loaded nanocarriers for combination therapy have been reported [12,13]. Most of the current studies focused on controlling the drugs release into the tumor targets [14–16], however, it is still ambiguous whether both drugs could be co-delivered into the same tumor cells. Thus, it would be pertinent to verify the delivery of different drugs into one cell, which would guarantee the optimized synergistic effect. Furthermore, most combination studies are based on the selection of the drugs derived from laboratory research, while

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lacking the clinical evidence. Therefore, appropriate drug combinations based on the clinical guidelines would have broader application prospects [17].

Among the nanocarriers, liposomes have been widely studied with promising application prospects [18,19]. For example, CPX-1 (Irinotecan plus Floxuridine) and CPX-351 (Cytarabine plus Daunorubicin), developed by Celator Pharmaceuticals Inc., have reached Phase II and Phase III clinical trials, respectively [20,21]. Liposomes can control the synchronous release of the drugs; improve the drug accumulation in tumor tissue by EPR effect; as well as reduce the toxicity of the drug to normal tissues [19,22]. Besides, liposomes have hydrophilic core which can encapsulate hydrophilic drugs, making them good carriers for co-delivery of oxaliplatin and irinotecan hydrochloride, since both these drugs are hydrophilic.

In the present study, oxaliplatin and irinotecan hydrochloride were co-loaded into the liposomes, followed by *in vitro* and *in vivo* evaluations. The co-loaded liposomes were designed to control the synchronous release of the drugs and hence enhance the anti-tumor therapeutic effect. The *in vitro* release properties were accessed using dialysis bag with diffusion method, and *in vitro* cytotoxicity study was tested in CT-26 and HCT-116 cells using MTT assay. Real-time near infrared fluorophore (NIRF) imaging was applied to monitor the fate of the liposomes *in vivo*, and cyro-sections were observed to evaluate the *in vivo* co-delivery ability of the liposomes into the tumor. In addition, *in vivo* anti-tumor efficacy was evaluated in CT-26 bearing BALB/c mice. The tissue toxicities of different formulations were studied by histological examination. To our knowledge, this is the first study on co-delivery of oxaliplatin and irinotecan hydrochloride for combination therapy.

2. Materials and methods

2.1. Materials

Oxaliplatin (OXA) was purchased from Boyuan Pharmaceutical Co., Ltd. (Jinan, China). Irinotecan hydrochloride (IRI) was purchased from Knowshine Pharmachemicals Inc. (Shanghai, China). Egg phospholipid was purchased from AVT Pharmaceutical Technology Co., Ltd. (Shanghai, China). Cholesterol was purchased from Sigma-Aldrich (St. Louis, MO, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) is the product of Solarbio (Shanghai, China). All other reagents were of analytical purity grade or higher, obtained commercially.

2.2. Cell cultures

Murine colon carcinoma cells (CT-26) and human colon cancer cells (HCT-116) were purchased from Chinese Academy of Sciences (China). Both cell lines were cultured in RPMI-1640 medium, supplemented with 10% FBS, 1% penicillin and 1% streptomycin at 37 °C in an environment containing 5% CO_2.

2.3. Animals

Female BALB/c mice (weight: 18–22 g) were supplied by the Medical Animal Test Center of Shandong University (Jinan, China). The animals were fed with a standard diet and allowed water ad libitum. All experiments were carried out in compliance with the Animal Management Rules of the Ministry of Health of the People's Republic of China (document number 55, 2001) and the Animal Experiment Ethics Review of Shandong University.

2.4. Synergistic effect between oxaliplatin and irinotecan

The synergistic effect of two drugs was verified using MTT assay in CT-26 cells. Both Gaddum model and Chou-Talalay method were applied to analyze the synergism. For the description of Gaddum model please refer to the Supplementary data. For Chou-Talalay method, oxaliplatin and irinotecan hydrochloride solutions were mixed into desired molar ratios, *i.e.* 1:0, 3:1, 1.5:1, 1:1, 1:1.5, 1:3 and 0:1. Cells were seeded in 96-well plates at a density of 5000 per well in 150 μL of RPMI-1640 medium. After overnight incubation, the cells were treated with different drug concentrations and incubated for 48 h, the concentration of the solution based on oxaliplatin ranged from 0.05 μM to 500 μM. Then 20 μL of MTT (5 mg/mL) was added to each well and incubated for another 4 h. The cell plates were centrifuged at 3000 rpm for 10 min and the culture medium was discarded, 150 μL of dimethyl sulphoxide (DMSO) was added to dissolve formazan crystals. The absorbance of the obtained DMSO solution was measured at a test wavelength of 570 nm with a microplate reader (Model 680, BIO-RAD, USA). The relative cell viability (%) was calculated by Eq. (1):

$$Cell\ \ viability(\%) = {A_{sample}}/{A_{control}} \times 100\% \quad (1)$$

where $A_{control}$ was the absorbance of negative control.

According to the cell viability values, concentration inhibiting half of the cells (IC_{50}) was calculated by SPSS software. The synergistic effect was evaluated by the combination index (*CI*) assay [14,23]. *CI* values were calculated based on the results of IC_{50} values using Chou-Talalay method [23].

$$CI = \frac{D_1}{D_{m1}} + \frac{D_2}{D_{m2}} \quad (2)$$

where D_1 and D_2 are the doses of drugs 1 and 2 that in combination produce 50% inhibition of cells and D_{m1} and D_{m2} are the doses of the drugs at which the drugs have the same effect when administered singly. The *CI* values lower than, equal to, and higher than 1 denote synergism, additivity and antagonism, respectively.

2.5. Preparation of liposomes

The preparation of oxaliplatin-loaded liposomes (OXA-Lip) and irinotecan-loaded liposomes (IRI-Lip) was described in Supplementary data.

Oxaliplatin and irinotecan co-loaded liposomes (OXIR-Lip) were prepared using EI method followed by ammonium sulphate gradient method [24–26]. Oxaliplatin and ammonium sulphate were dissolved in 4 mL of 5% glucose solution. Separately, 120 mg egg phosphatidylcholine and 30 mg cholesterol were dissolved in 1 mL ethanol. The ethanolic solution was added dropwise into the aqueous phase at 60 °C. A rotary evaporator was employed to remove ethanol and oxaliplatin-loaded liposomes formed subsequently. Liposomes were dialyzed in 600 mL of 5% glucose to remove free oxaliplatin and

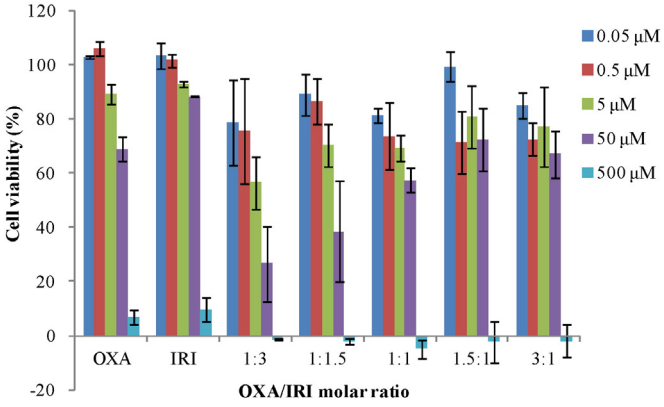

Fig. 1. Cell viabilities of OXA/IRI mixtures at different molar ratios in CT-26 cells. The concentration in each combined solution was based on OXA, while the concentration of IRI was depended on the OXA/IRI molar ratios.